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(54) Title: METHOD FOR REDUCING PHYTATE IN CANOLA MEAL USING GENETIC MANIPULATION INVOLVING MYO-INOSITOL 1-PHOSPHATE SYNTHASE GENE

(57) Abstract: A plant, multicellular fragment of said plant or seed of said plant transformed with a nucleotide sequence of SEQ ID NO 1 or an allelic variant or a fragment thereof or a genetic equivalent thereof according to the degeneracy of the genetic code coding for a peptide having a Brassica myo-inositol 1-phosphate synthase activity, said plant, multicellular fragment or seed having reduced myo-inositol 1-phosphate synthase activity when compared with an equivalent untransformed plant, multicellular fragment or seed, such that there is reduced phytate present in the plant, multicellular fragment or seed. The invention also provides a method for reducing phytate in Brassica, which method comprises growing a Brassica plant comprising one of a myo-inositol 1-phosphate synthase antisense sequence and a myo-inositol 1-phosphate synthase cosuppression sequence thereby yielding a reduced amount of myo-inositol 1-phosphate synthase and consequently reduced phytate in said Brassica.

**METHOD FOR REDUCING PHYTATE IN CANOLA MEAL USING GENETIC  
MANIPULATION INVOLVING MYO-INOSITOL 1-PHOSPHATE SYNTHASE GENE**

**BACKGROUND OF THE INVENTION**

5

The use of canola meal as an acceptable protein source in the animal feed industry is severely limited by the presence in the meal of undesirable seed contents such as glucosinolates, phytates, phenolics and hull. Phytate is a significant component of canola seeds, comprising up to 10% by weight of the seed. Phytic acid is the hexaphosphate derivative of myo-inositol. The presence of phytate has been linked to such symptoms as loss of appetite, reduced litter size and increase in the number of stillborn pups in rats. These effects have been attributed to the zinc-binding ability of phytic acid. The reduction of phytate in canola protein preparations has, to date, been difficult. Hence modification of its biosynthetic pathway to reduce its accumulation and enhance protein or oil synthesis in its place would be very significant in terms of the economic value of canola.

**SUMMARY OF THE INVENTION**

An aim of the present invention is to limit the utilization of myo-inositol as a starting material for phytic acid synthesis. This is complicated by the fact that myo-inositol is a crucial biological substrate, the presence of which is essential for the growth and multiplication of all living cells. In plants, for example, in addition to its participation in cell wall biogenesis, where myo-inositol furnishes a carbon source for uronides and pentoses, it is also present in phosphoinositides of plant cell membranes, as well as other complex plant lipids including glycoposphoceramides. Additionally, in some of its phosphorylated forms it acts as important second messengers in signal transduction pathways in eukaryotes. It is also a precursor of other naturally occurring inositol isomers and many of these as well as myo-inositol are distributed as methyl ethers in a species specific pattern throughout the plant kingdom.

30 In view of the vital role myo-inositol plays in plants, limiting its supply to the cell can be expected to promote reorganization of priorities within the cell, with possibly unforeseen consequences. Myo-inositol pathways leading to critical cell components or functions may be expected to proceed at the expense of other pathways that are of no direct and immediate consequence to the well-being of the plant during its life cycle. Phytic acid synthesis is an example of such a "futile" pathway since phytic acid is a storage substance that does not take

part in any of the essential pathways during plant growth and development. It therefore seemed possible to us that, by limiting the availability of myo-inositol 1-phosphate synthase, lesser amounts of glucose 6-phosphate would be converted to myo-inositol thereby leading to a lower rate of phytic acid synthesis. It can be seen that a difficult balance has to be struck  
5 between over-limiting the production of myo-inositol thereby threatening critical pathways and under-limiting the production of myo-inositol with little consequent economic benefit.

The basic approach to limiting the rate of conversion of glucose 6-phosphate to myo-inositol 1-phosphate in Brassica napus was to prepare a mRNA transcript for the enzyme responsible for this step from B. napus and then to introduce a recombinant version of this  
10 gene into Brassica plants by, for example, Agrobacterium-mediated transformation in two different orientations (sense, for cosuppression, and antisense). Two particular types of constructs were produced containing either the 35S promoter or the seed-specific napin promoter. Integration of the construct into the genome was confirmed by Southern blot analysis. Phytic acid analysis showed reduction in levels of around 30% to 50% for antisense  
15 and cosuppression transgenic plants.

The invention provides a nucleotide sequence of SEQ ID NO 1 or an allelic variant or a fragment thereof or a genetic equivalent thereof according to the degeneracy of the genetic code coding for a peptide having a Brassica myo-inositol 1-phosphate synthase activity.

In preferred embodiments the nucleotide sequence, variant or fragment of the invention  
20 is in combination with a promoter sequence in reading frame alignment (preferably antisense) therewith.

The invention also provides a myo-inositol 1-phosphate synthase-active peptide sequence encoded by the nucleotide sequence of the invention. The invention also provides cells (preferably Brassica, especially B. napus cells) transformed with a nucleotide sequence  
25 of the invention. The invention further provides plants, multicellular fragments of such plants and seeds of such plants transformed with a nucleotide of the invention, the plant, multicellular fragment or seed having reduced myo-inositol 1-phosphate synthase activity such that there is reduced phytate present in the plant, multicellular fragment or seed. The multicellular fragment is preferably in the form of a seed meal.

30 The invention also provides a method for reducing phytate in Brassica which comprises limiting the availability of myo-inositol 1-phosphate synthase in said Brassica with one of one of a myo-inositol 1-phosphate synthase antisense sequence and a myo-inositol 1-phosphate synthase cosuppression sequence to give a reduced amount of translatable myo-inositol 1-phosphate synthase thereby reducing phytate in said Brassica. Preferably the Brassica is

antisense or  
cosuppression

Brassica napus. The invention also provides a method for reducing phytate in Brassica, which method comprises growing a Brassica plant comprising one of a myo-inositol 1-phosphate synthase antisense sequence and a myo-inositol 1-phosphate synthase cosuppression sequence thereby yielding a reduced amount of myo-inositol 1-phosphate synthase and consequently reduced phytate in said Brassica.

#### DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO: 1 shows the nucleotide sequence of a myo-inositol 1-phosphate synthase gene of Brassica napus.

SEQ ID NO: 2 show the amino acid sequence of myo-inositol 1-phosphate synthase of Brassica napus.

SEQ ID NO: 3 shows the myo-inositol 1-phosphate synthase right primer used in the examples.

SEQ ID NO: 4 shows the myo-inositol 1-phosphate synthase left primer used in the examples.

SEQ ID NO: 5 shows a sequence comprising the myo-inositol 1-phosphate synthase (mips) promoter sequence used in the examples.

#### DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of the Brassica napus myo-inositol 1-phosphate synthase of the invention.

#### DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "functional fragments" when used to modify a specific gene or gene product means a less than full length portion of the gene or gene product which retains substantially all of the biological function associated with the full length gene or gene product to which it relates. To determine whether a fragment of a particular gene or gene product is a

functional fragment, fragments are generated by well-known nucleolytic or proteolytic techniques or by the polymerase chain reaction and the fragments tested for the described biological function.

As used herein, a coding sequence is "operably linked to" another coding sequence when  
5 RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequence is ultimately processed to produce the desired protein.

As used herein, "recombinant" polypeptides refer to polypeptides produced by  
10 recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

As used herein, a "replicon" is any genetic element (e.g., plasmid, chromosome, virus)  
that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication  
15 under its own control.

As used herein, a "vector" is a replicon, such as a plasmid, phage, or cosmid, to which  
another DNA segment may be attached so as to bring about the replication of the attached  
segment.

As used herein, a "reference" gene refers to the wild type gene sequence of the invention  
20 and is understood to include the various sequence polymorphisms that exist, wherein nucleotide substitutions in the gene sequence exist, but do not affect the essential function of the gene product.

As used herein, a "mutant" gene refers sequences different from the reference gene  
wherein nucleotide substitutions and/or deletions and/or insertions result in perturbation of the  
25 essential function of the gene product.

As used herein, a DNA "coding sequence of" or a "nucleotide sequence encoding" a  
particular protein, is a DNA sequence which is transcribed and translated into a polypeptide  
when placed under the control of appropriate regulatory sequences.

As used herein, a "promoter sequence" is a DNA regulatory region capable of binding  
30 RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding  
sequence. For purposes of defining the present invention, the promoter sequence is bound at its  
3' terminus by a translation start codon (e.g., ATG) of a coding sequence and extends upstream  
(5' direction) to include the minimum number of bases or elements necessary to initiate  
transcription at levels detectable above background. Within the promoter sequence will be found

a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35  
5 consensus sequences.

As used herein, DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers and the like, which collectively provide for the expression (i.e., the transcription and translation) of a coding sequence in a host cell.

10 As used herein, a control sequence "directs the expression" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

As used herein, a "host cell" is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous DNA sequence.

15 As used herein, a cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eukaryotic cells, a stably transformed or transfected  
20 cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

As used herein, "transfection" or "transfected" refers to a process by which cells take up  
25 foreign DNA and integrate that foreign DNA into their chromosome. Transfection can be accomplished, for example, by various techniques in which cells take up DNA (e.g., calcium phosphate precipitation, electroporation, assimilation of liposomes, etc.) or by infection, in which viruses are used to transfer DNA into cells.

As used herein, a "target cell" is a cell that is selectively transfected over other cell types  
30 (or cell lines).

As used herein, a "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

As used herein, a "heterologous" region of a DNA construct is an identifiable segment of

DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a gene, the gene will usually be flanked by DNA that does not flank the gene in the genome of the source. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

An aspect of the present invention is isolated polynucleotides encoding a protein including substantially similar sequences and functional fragments. Isolated polynucleotide sequences are substantially similar if they are capable of hybridizing under moderately stringent conditions to SEQ ID NO:1 or they encode DNA sequences which are degenerate to SEQ ID NO:1 or are degenerate to those sequences capable of hybridizing under moderately stringent conditions to SEQ ID NO:1.

Moderately stringent conditions is a term understood by the skilled artisan and has been described in, for example, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd edition, Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989). An exemplary hybridization protocol using moderately stringent conditions is as follows. Nitrocellulose filters are prehybridized at 65.degree. C. in a solution containing 6.times. SSPE, 5.times. Denhardt's solution (10 g Ficoll, 10 g BSA and 10 g polyvinylpyrrolidone per litre solution), 0.05% SDS and 100 µg/ml tRNA. Hybridization probes are labelled, preferably radiolabelled (e.g., using the Bios TAG-IT.RTM. kit). Hybridization is then carried out for approximately 18 hours at 65.degree. C. The filters are then washed twice in a solution of 2.times. SSC and 0.5% SDS at room temperature for 15 minutes. Subsequently, the filters are washed at 58.degree. C., air-dried and exposed to X-ray film overnight at -70.degree. C. with an intensifying screen.

Degenerate DNA sequences encode the same amino acid sequence as SEQ ID NO:2 or the proteins encoded by that sequence capable of hybridizing under moderately stringent conditions to SEQ ID NO:1, but have variation(s) in the nucleotide coding sequences because of the degeneracy of the genetic code. For example, the degenerate codons UUC and UUU both code for the amino acid phenylalanine, whereas the four codons GGX all code for glycine.

Alternatively, substantially similar sequences are defined as those sequences in which about 70%, preferably about 80% and most preferably about 90%, of the nucleotides or amino acids match over a defined length of the molecule. As used herein, substantially similar refers to the sequences having similar identity to the sequences of the instant invention. Thus nucleotide sequences that are substantially the same can be identified by hybridization or by sequence

comparison. Protein sequences that are substantially the same can be identified by techniques such as proteolytic digestion, gel electrophoresis and/or microsequencing.

Embodiments of the isolated polynucleotides of the invention include DNA, genomic DNA and RNA, preferably of Brassica origin. A method for isolating a nucleic acid molecule encoding  
5 a protein is to probe a genomic or cDNA library with a natural or artificially designed probe using art recognized procedures. See, e.g., "Current Protocols in Molecular Biology", Ausubel et al. (eds.) Greene Publishing Association and John Wiley Interscience, New York, 1989, 1992. The ordinarily skilled artisan will appreciate that SEQ ID NO:1 or fragments thereof comprising at least 15 contiguous nucleotides are particularly useful probes. It is also appreciated that such  
10 probes can be and are preferably labelled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include, but are not limited to, radioisotopes, fluorescent dyes or enzymes capable of catalysing the formation of a detectable product. The probes would enable the ordinarily skilled artisan to isolate complementary copies of genomic DNA, cDNA or RNA polynucleotides encoding proteins from Brassica or other plant sources  
15 or to screen such sources for related sequences, e.g., additional members of the family, type and/or subtype, including transcriptional regulatory and control elements as well as other stability, processing, translation and tissue specificity-determining regions from 5' and/or 3' regions relative to the coding sequences disclosed herein, all without undue experimentation.

Another aspect of the invention is functional polypeptides encoded by the polynucleotides  
20 of the invention. An embodiment of a functional polypeptide of the invention is the Brassica protein having the amino acid sequence set forth in SEQ ID NO:2.

Another aspect of the invention is a method for preparing essentially pure Brassica protein. Yet another aspect is the Brassica protein produced by the preparation method of the invention. This protein has the amino acid sequence listed in SEQ ID NO:2 and includes variants with a  
25 substantially similar amino acid sequence that have the same function. The proteins of this invention can be made by recombinant genetic engineering techniques by culturing a recombinant host cell containing a vector encoding the polynucleotides of the invention under conditions promoting the expression of the protein and recovery thereof.

The isolated polynucleotides, particularly the DNAs, can be introduced into expression  
30 vectors by operatively linking the DNA to the necessary expression control regions, e.g., regulatory regions, required for gene expression. The vectors can be introduced into an appropriate host cell such as a prokaryotic, e.g., bacterial, or eukaryotic, e.g., yeast or plant cell by methods well known in the art. See Ausubel et al., supra. The coding sequences for the desired proteins, having been prepared or isolated, can be cloned into any suitable vector or

replicon. Numerous cloning vectors are known to those of skill in the art and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include, but are not limited to, the bacteriophage lambda (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pGEX4T-3 (E. coli), pKT230  
5 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces) and YCp19 (Saccharomyces). See generally, "DNA Cloning": Vols. I & II, Glover et al. ed. IRL Press Oxford (1985) (1987); and T. Maniatis et al. ("Molecular Cloning" Cold Spring Harbor  
10 Laboratory (1982).

The gene can be placed under the control of control elements such as a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator, so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing the expression construct. The coding sequence may or may not contain a  
15 signal peptide or leader sequence. The proteins of the present invention can be expressed using, for example, the E. coli tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Pat. Nos. 4,431,739; 4,425,437 and 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which  
20 allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art. Exemplary are those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound or to various temperature or metabolic conditions. Other types of regulatory elements may also be present in the vector, for  
25 example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences, i.e., RNA polymerase which binds to the DNA  
30 molecule at the control sequences transcribes the coding sequence. Modification of the sequences encoding the particular antigen of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior

to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to produce mutants or analogues of Brassica protein.

5 Mutants or analogues may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; "DNA Cloning," Vols. I and II, supra; and "Nucleic Acid Hybridization", supra.

10 Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. Preferred plant cells include Brassica cells. If the expression system secretes the protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates  
15 or recovered from the cell membrane fraction. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify proteins of the present invention is by constructing gene libraries, using the resulting clones to transform E. coli and pooling and screening individual colonies.

20 The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis on an automated peptide synthesizer, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art.

Another aspect of the invention is antisense oligonucleotides comprising a sequence which  
25 is capable of binding to the polynucleotides of the invention. Synthetic oligonucleotides or related antisense chemical structural analogs can be designed to recognize, specifically bind to and prevent transcription of a target nucleic acid encoding protein by those of ordinary skill in the art. See generally, Cohen, J. S., Trends in Pharm. Sci., 10, 435(1989) and Weintraub, H. M., Scientific American, January (1990) at page 40. By "antisense" RNA is meant a complementary  
30 RNA sequence that binds to and blocks the transcription of a naturally occurring sense messenger RNA molecule.

By "cosuppression" is meant the phenomenon of native gene silencing as a result of attempting to over-express the same gene, by recombinant DNA, in its original host plant (from which the gene has been isolated). In the case of this invention the myo-inositol 1-phosphate (mips) gene

was isolated from Brassica napus and was re-introduced back into B. napus by Agrobacterium tumefaciens transformation.

Defining appropriate hybridization conditions is within the skill of the art. See, e.g., "Current Protocols in Mol. Biol." Vol. I & II, Wiley Interscience. Ausbel et al. (eds.) (1992).

5 Probing technology is well known in the art and it is appreciated that the size of the probes can vary widely but it is preferred that the probe be at least 15 nucleotides in length. It is also appreciated that such probes can be and are preferably labelled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioisotopes, fluorescent dyes or enzymes capable of catalysing the formation of a detectable  
10 product. As a general rule, the more stringent the hybridization conditions the more closely related genes will be that are recovered.

Another aspect of the invention is transgenic, non-Brassica plants capable of expressing the polynucleotides of the invention in any cell. Transgenic, non-Brassica plants may be obtained by transfecting with the polynucleotides of the invention. The resultant transgenic plant may be  
15 used as a model for the study of gene function or for producing large amounts of protein for screening or crystallography purposes. Particularly useful transgenic plants are those which display a detectable phenotype associated with the expression of the protein.

### Experimental Results

20

#### a) Cloning of myo-inositol 1-phosphate synthase (MIPS) gene from B. napus:

A cDNA copy of MIPS gene was isolated from B. napus developing seed by the RT-PCR method. RT-PCR was conducted by synthesizing 1st strand cDNA of MIPS using the 1st strand cDNA  
25 synthesis kit (Boehringer Mannheim). Briefly, 1 µg of total RNA from B. napus developing seeds was added to 0.5 ml tube containing MIPS-right primer (5' AAAAATCTAGAGTGAACACTTGTATTCCAAGATCA 3'), 1x RT (reverse transcriptase) buffer, the four dNTPs, and water. The mixture was incubated at 25° C for 10 min and was then placed on ice. Subsequently, 20 U of RT (reverse transcriptase) was added, mixed very well. The  
30 reaction was initiated by incubating the tube at 42° C for 60 min. Finally, heating at 95° C for 10 min inactivated the RT enzyme. The product is the 1st strand of MIPS gene.

For PCR amplification, 5 µL of 1st strand cDNA solution was added to 0.5 ml tube containing 1x Vent polymerase buffer, the four dNTPs, the MIPS-left primer (5' AAAAACC CGGATGATCGAGAGCTTCAAAGTC 3'), the right primer, and water. The

reaction was initiated by addition of 1 U of Vent DNA polymerase, mixed very well and placed in a DNA thermal cycler (Perkin Elmer Cetus). Heating at 94° C for 2 min followed by 35 cycle each of which includes heating at 94° C for 1 min, annealing at 52° C for 1 min and extension at 72° C for 3 min.

- 5 The left and right primer (containing *XbaI* and *SmaI* site respectively) were synthesized according to the published MIPS DNA sequence of Arabidopsis thaliana (GenBank accession number U04876).

At the end of the PCR run, 5 µL of the reaction solution was loaded on 1.2% Agarose gel. The PCR product was purified by PCR purification kit (Promega) and was then digested with both *XbaI* and *SmaI*. The digested DNA was loaded on gel and the 1.7 Kb fragment was eluted and purified. The purified fragment was then cloned into pSPORT1 (GIBGOBRL) pre-cut with both *XbaI* and *SmaI*. The clone containing the insert was subjected to full length DNA sequencing.

Total RNA was extracted from plant tissue by using RNeasy plant total RNA kit (Qiagen).

#### 15 b) DNA sequence analysis

DNA sequencing was performed by the DNA Services Lab at PBI using ABI Prism Dye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase and following the company's protocol (Perkin Elmer).

20

These primers as well as those for sequencing were synthesized in an Applied Biosystems DNA synthesizer.

#### c) Cloning of MIPS gene in pBI121

25

The MIPS cDNA was cloned under CamV35S or B. napus napin promoters in both orientations (sense or anti-sense) in the plant vector pBI121 (from Clontech). The constructs were transferred into Agrobacterium tumefaciens strain CV3101: pMP90RK by electroporation. The transformants were grown on kanamycin-containing plates and then screened by PCR

- 30 analysis for the presence of the construct. The positive transformants were used for Brassica napus transformation. Plant transformation was conducted according to Maloney et al, 1989 (Maloney, N.M., Walker, J.M., and Sharma, K.K. 1989, Plant Cell Reports 8, 238)

**d) Isolation of the MIPS genomic promoter**

The genomic MIPS promoter was isolated from B. napus by PCR with a primer designed to read beyond the 5' end of the MIPS gene. The DNA sequence of this promoter probably  
5 extends much beyond the promoter itself. This promoter would be useful for targeting the expression of foreign genes to the same location and at the same time (spatial- and temporal-mode of expression). In this way more precise deactivation of MIPS gene can be achieved. Also, it can be used in any experiments where the desired trait must accompany the expression of the native MIPS gene. The sequence including the MIPS promoter(3795 bp) from B. napus  
10 that we cloned is shown in SEQ ID NO:5.

**e) Plant materials:**

Brassica napus, Var. Westar, the control and transgenic , plants were grown in a growth-  
15 chamber under a 16hr. light cycle at 20°C, followed by 8hr. darkness at 15°C. Phytate levels were measured and compared for control and transgenic plants. Reductions of 30% to 50% in phytate levels were found.

We claim:

1. A nucleotide sequence of SEQ ID NO 1 or an allelic variant or a fragment thereof or a genetic equivalent thereof according to the degeneracy of the genetic code  
5 coding for a peptide having a Brassica myo-inositol 1-phosphate synthase activity.
2. A nucleotide sequence, variant or fragment according to claim 1 in combination with a promoter sequence in reading frame alignment therewith.
- 10 3. A nucleotide sequence, variant or fragment according to claim 1 in combination with a promoter sequence in antisense reading frame alignment therewith.
4. A myo-inositol 1-phosphate synthase-active peptide sequence encoded by the nucleotide sequence of claim 1.
- 15 5. A cell transformed with a nucleotide according to claim 1.
6. A Brassica cell transformed with a nucleotide according to claim 1.
- 20 7. A plant, multicellular fragment of said plant or seed of said plant transformed with a nucleotide according to claim 1, said plant, multicellular fragment or seed having reduced myo-inositol 1-phosphate synthase activity when compared with an equivalent untransformed plant, multicellular fragment or seed, such that there is reduced phytate present in the plant, multicellular fragment or seed.
- 25 8. A multicellular fragment according to claim 7 in the form of seed meal.
9. A Brassica napus myo-inositol 1-phosphate synthase promoter sequence.
- 30 10. A method for reducing phytate in Brassica, which method comprises growing a Brassica plant comprising at least one of a myo-inositol 1-phosphate synthase

antisense sequence and a myo-inositol 1-phosphate synthase cosuppression sequence thereby yielding a reduced amount of myo-inositol 1-phosphate synthase and consequently reduced phytate in said Brassica.

- 5 11. A method according to claim 10 wherein said Brassica is Brassica napus.
12. A method according to claim 10 wherein said Brassica comprises a myo-inositol 1-phosphate synthase antisense sequence.
- 10 13. A method according to claim 10 wherein said Brassica comprises a myo-inositol 1-phosphate synthase cosuppression sequence.

1/1

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Fig. 1

## SEQUENCE LISTING

<110> Georges, Fawzy  
Hussain, Atta A  
5 Keller, Wilfred A

<120> Method for Reducing Phytate in Canola Meal Using  
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<130> Method for reducing phytate in canola

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00612

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N15/61 C12N15/11 C12N9/90 A23K1/165

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! ACCESSION NO: U66307, 30 August 1986 (1986-08-30) HUSSAIN, A., ET AL.: XP002148805	1,4-8
Y	the whole document  -& DATABASE GENBANK 'Online! GI:1513228 GENPEPT, 29 September 1996 (1996-09-29) HUSSAIN, A., ET AL.: XP002148806 abstract  -/-	2,3,8, 10-13

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

29 September 2000

Date of mailing of the international search report

17/10/2000

Name and mailing address of the ISA

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Authorized officer

Maddox, A

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00612

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Y	page 5, line 9 - line 14  page 6, line 25 - page 7, line 2 page 7, line 9 - line 11 page 7, line 19 - line 20 page 8, line 1 - line 27 page 9, line 4 - line 10 page 14, line 16 - line 27 page 15, line 7 - line 22 page 17, line 21 - line 28 page 27, line 1 - line 8; claims 1-32; figures 2,4,8	2,3,8, 10-13
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-/-

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00612

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 91 14782 A (MOGEN INT ;GIST BROCADES NV (NL)) 3 October 1991 (1991-10-03) example 9	1-13
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